Biochemical Pharmacology, Vol. 31, No. 7, pp. 1273–1277, 1982.
Printed in Great Britain.

0006-2952/82/071273–05 $03.00/0

FUNCTIONAL HETEROGENEITY OF UDP-GLUCURONOSYLTRANSFERASE ACTIVITIES IN C57BL/6 AND DBA/2 MICE

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(*Received* 21 *August* 1981; *accepted* 22 *October* 1981)

Abstract—Functional heterogeneity of liver microsomal UDP–glucuronosyltransferase activities towards 1-naphthol, 4-methylumbelliferone or 3-hydroxybenzo(a)pyrene (UDP–GT_1 activities) and morphine or 4-hydroxybiphenyl (UDP–GT_2 activities) was studied in two inbred strains of mice which are genetically responsive (C57BL/6) or non-responsive (DBA/2) to 3-methylcholanthrene-induction of drug metabolizing enzymes. 3-Methylcholanthrene preferentially induced UDP–GT_1 activities in C57BL/6 mice. Phenobarbital, however, at low doses (50 mg/kg), selectively induced UDP–GT_2 activities. Higher doses of phenobarbital (80 mg/kg) induced both UDP–GT_1 and UDP–GT_2 activities. In DBA/2 mice 3-methylcholanthrene-induction of UDP-glucuronosyltransferase activities was not detectable whereas enzyme induction by phenobarbital appeared to be unimpaired. UDP–GT_1 activities were ubiquitously detectable in mouse tissues whereas appreciable UDP–GT_2 activities were only found in liver and small intestinal mucosa. UDP–GT_1 (1-naphthol as substrate) was not inhibited by morphine suggesting different active sites for the conjugation of these substrates. The results suggest the presence of at least two functionally different forms of UDP–glucuronosyltransferase in mice. In conjunction with the results of Owens (*J. biol. Chem.* **252**, 2827 (1977)) it is evident that one of these enzyme forms is regulated by the Ah locus.

Glucuronidation is quantitatively the most important pathway in the elimination of endogenous compounds such as bilirubin, thyroxine and steroid hormones, and in phase 2 of drug metabolism [1]. Moreover this conjugation reaction, together with others, often prevents accumulation of toxic nucleophilic metabolites of carcinogens, e.g., of benzo(a)pyrene phenols which are cytotoxic [2] and can be further oxidized to ultimate DNA binding and mutagenic metabolites [3–6]. The reaction is catalysed by microsomal UDP–glucuronosyltransferase (GT, EC 2.4.1.17) which probably consists of a family of closely related enzyme forms with different but overlapping substrate specificities [1]. Recently certain glucuronidation reactions could be classified on the basis of their preferential inducibility in rat liver by 3-methylcholanthrene (3-MC) or phenobarbital [7, 8], prototypes of two different classes of inducing agents of drug metabolizing enzymes [9]. The same reactions can also be distinguished by their different perinatal development [10] and tissue distribution [11] in the rat. Enzymes catalysing either 3-MC-inducible or phenobarbital-inducible reactions, operationally termed GT_1 and GT_2, respectively, could be separated and purified to apparent homogeneity [12]. On the basis of the above criteria GT_1 substrates are, e.g., 1-naphthol, 4-methylumbelliferone and 3-hydroxybenzo(a)pyrene. Morphine, 4-hydroxybiphenyl and chloramphenicol are termed GT_2 substrates. Bilirubin [13] or oestrone [14] may be conjugated by yet other forms of GT.

It was the purpose of the present study to investigate whether the properties of GT (substrate specificity and inducibility), which led to the distinction between rat liver GT_1 and GT_2 activities, were also found in other species. We studied two inbred strains of mice, C57BL/6 and DBA/2, which are genetically responsive and non-responsive to 3-MC-type inducers of cytochrome P_1-450* dependent aryl hydrocarbon hydroxylase (AHH), respectively [15]. Defective inducibility was attributed to a genetic defect of the Ah locus, the Ah^d allele. The murine Ah locus has been found to control 3-MC-type induction of several drug metabolizing enzyme activities in the liver and in virtually all nonhepatic tissues examined. In genetic experiments with C57BL/6 and DBA/2 mice it was recently found that 3-MC-induction of GT (4-methylumbelliferone as substrate) co-segregates with AHH induction [19]. Coordinate regulation of these two functionally related enzymes is probably mediated by a common cytosolic receptor protein for 3-MC-type inducers [16, 20]. This receptor is viewed as the major Ah regulatory gene product. In addition to their importance for the elucidation of the molecular mechanism of 3-MC-type induction, responsive and non-responsive inbred strains of mice have also been of great importance for studies on the biological consequences of inducibility [15].

MATERIALS AND METHODS

Chemicals were obtained from the following

* Mouse liver cytochrome P_1-450 has been defined arbitrarily by Nebert [15] as the 3-MC-inducible form of P-450 most closely associated with 3-MC-inducible aryl hydrocarbon hydroxylase activity. P_1-450 is not to be confused with mouse liver 3-MC-inducible P-448 or with rat liver P_1-450 [17] which in the latter species is identical with P-448 or P-450_c [18].

sources: *N*-[^{14}C-methyl]morphine–HCl from Amersham–Buchler (Braunschweig, F.D.R.); Brij 58, polyoxyethylene(20)acetylether, a gift from Atlas (Essen, F.D.R.); 3-hydroxybenzo(a)pyrene provided by the ITT Research Institute (Chicago, IL).

Homozygous C57BL/6J (Ah^bAh^b) and DBA/2J (Ah^dAh^d) mice were a generous gift of Dr. J. E. Gielen, Laboratoire de Chimie Médicale, Institut de Pathologie, B-4000 Sart-Tilman par Liège, Belgium. Both strains are maintained in our animal house, and fed a standard diet containing 20% protein (Altromin, Lage-Lippe, F.D.R.).

Treatment with inducing agents. Male animals between 4–6 weeks (18–24 g) were used.

Treatment with 3-MC. Animals received one i.p. injection of 3-MC (80 or 200 mg/kg, dissolved in 0.2 ml olive oil). Controls received 0.2 ml olive oil only. Animals were killed 4 days after treatment. No significant difference was noted between untreated animals and controls receiving olive oil.

Treatment with phenobarbital. Sodium phenobarbital (40–60 mg/kg) was injected daily for 4 days, or a dose of 80 mg/kg was administered once i.p., followed by 0.1% in drinking water for 4 days. Under the latter conditions 30% of the animals died.

Preparation of tissue homogenates and microsomes. Mouse livers were perfused with 0.9% NaCl while the animals were under ether anaesthesia. After killing, other organs were excised and immediately chilled with ice-cold 0.9% NaCl. Homogenates and microsomes were prepared with 0.25 M sucrose containing 10 mM Tris–HCl, pH 7.4, as described for rat liver [21]. Liver microsomes could be stored at −20° up to 1 week without loss of enzyme activity.

Pieces of duodenum and small intestine were rinsed extensively with 0.9% NaCl containing 10^{-4} M phenylmethylsulfonyl fluoride, a protease inhibitor. The mucosa was scraped off with a spatula, and homogenates (10%, w/v) were prepared in 0.25 M buffered sucrose. Under these conditions some spontaneous activation of GT probably cannot be prevented, similar to observations with rat intestinal GT [22].

Skin homogenates. After killing the animals were shaved at the dorsal skin (5 × 3 cm) with an electrical clipper. The shaved areas were excised, subcutaneous fat and muscle was scraped off with a scalpel. The skin preparation was frozen in liquid nitrogen and powdered using a metal mortar and stored at −80°. For enzyme assays up to 5 mg of skin powder was weighed and suspended in the incubation mixture. Skin powder had to be weighed into the test tube directly instead of using homogenate fractions. It was recently found that, in contrast to aryl hydrocarbon hydroxylase, a considerable part of GT activity (70–80%) was found in the sediment when the skin homogenate was centrifuged at 500 *g* (unpublished). Using skin powder suspensions 1-naphthol-GT can be activated 2-fold by the addition of Brij 58. However it is likely that some spontaneous activation occurred during the procedure adopted to homogenize the tissue.

Protein. Protein was determined according to Lowry *et al.* [23] using bovine serum albumin as protein standard.

Assays of UDP–glucuronosyltransferase. Enzyme activities towards various substrates were assayed by the following aglycone concentrations and by methods already described (unless specified): 0.5 mM 1-naphthol [21]; 0.5 mM 4-methylumbelliferone (see below); 0.05 mM 3-hydroxybenzo(a)pyrene [24]; 1.5 mM morphine [25]; 0.5 mM 4-hydroxybiphenyl ([12], modified as described below); 0.1 mM bilirubin [26]. With 4-hydroxybiphenyl as substrate, perchloric acid (0.5 M) instead of trichloroacetic acid [12] was used to stop the reaction. By this modification the fluorescence of the glucuronide is enhanced 3-fold without affecting background fluorescence. For reasons of standardization and comparison the assays were performed at 37° in the presence of 0.1 M Tris–HCl, pH 7.4, and 5 mM $MgCl_2$. Reactions were started by addition of 3 mM UDP-glucuronic acid.

Care was taken to achieve full enzyme activation and to perform the assay under conditions leading to linear reaction rates with time and protein concentration. The enzyme was fully activated by the addition of Brij 58 (1 mg/mg protein) except for bilirubin-GT which was activated by the addition of digitonin (0.15%, w/v; 3 mg/mg protein). For evaluation of the reliability of the enzyme assay we recommend to determine the latency characteristics of the enzyme preparation. When the enzyme has already been fully activated during isolation of microsomes, as in isolated intestinal microsomes [22], addition of detergent would lead to the inhibition of enzyme activity. Under our conditions liver microsomal 1-naphthol-GT activity in the 'native', UDP-*N*-acetylglucosamine-activated (3 mM) and Brij-58-activated state was 2.0 ± 1.0, 5.1 ± 1.2 and 26.5 ± 5.9 nmoles/min/mg protein, respectively. UDP-*N*-acetylglucosamine is probably a physiological activator of GT [27].

Our fluorescence assays are based on monitoring fluorescence of the respective glucuronides. They were carried out using a Perkin Elmer 650-10S fluorescence spectrophotometer. Fluorescence intensity was calibrated with quinine sulfate.

UDP-glucuronosyltransferase activity with 4-methylumbelliferone as substrate. 4-Methylumbelliferone (0.5 mM final concentration) was dissolved in dimethylsulfoxide and added to the standard incubation mixture (0.5 ml) specified above. With liver microsomes the assay usually contained 0.02 mg protein. The reaction was started by addition of 3 mM UDP–glucuronic acid and stopped after 2 and 5 min by addition of 0.5 M perchloric acid (0.5 ml). Excessive substrate was extracted with 2 ml chloroform. After centrifugation an aliquot of the water phase containing the glucuronide (0.5 ml) was mixed with 0.5 ml 1.6 M glycine/NaOH, pH 10.3. Fluorescence was measured at 365 nm with excitation at 315 nm. Calibration was performed with 4-methylumbelliferyl-β, D-glucuronide. Traces of the unconjugated substrate, showing maximal fluorescence at 445 nm with excitation at 365 nm, did not interfere with the fluorescence of the glucuronide.

Monooxygenase assays. AHH activity was determined by the method of Nebert and Gelboin [28]

Table 1. Differential induction of liver microsomal GT_1 and GT_2 activities at various doses of phenobarbital in C57BL/6 mice

Dose of phenobarbital (mg/kg, daily i.p., for 4 days)	UDP-glucuronosyltransferase activity* (nmoles/min/mg protein) 1-Naphthol	Morphine
—	24.4 ± 4.2	11.6 ± 1.5
40	25.1 ± 3.9	14.3 ± 3.0
50	25.4 ± 2.4	16.7 ± 2.3
60	28.8 ± 2.4	25.2 ± 4.6
80†	60.5 ± 4.9	25.5 ± 3.0

* Values represent the mean ±S.D. of 4 induction experiments.
† Once i.p., then 0.1% in drinking water.

which follows the formation of fluorescent phenols. It was calibrated with 3-hydroxybenzo(a)pyrene as substrate. Aminopyrine-*N*-demethylase was assayed as described [29]. Standard incubation mixtures contained 0.1 M Tris–HCl, pH 7.4, 0.5 mM NADP, 5 mM $MgCl_2$, 5 mM sodium isocitrate and 0.2 I.U. isocitrate dehydrogenase. In blanks the NADPH regenerating system was omitted.

Statistical evaluation of the results was done using Student's *t*-test.

RESULTS

Differential induction of UDP-glucuronosyltransferase activities by 3-methylcholanthrene or phenobarbital. Differential inducibility of various GT activities by phenobarbital was found to be dose-dependent in C57BL/6 mice (Table 1). Treatment with a dose of 60 mg/kg led to 2-fold increase of morphine-GT activity but not to a significant enhancement of 1-naphthol-GT activity. Toxic doses of phenobarbital (80 mg/kg, once i.p., then 0.1% in drinking water for 4 days) led to greater than 2-fold induction of both GT_1 and GT_2 activities. Hence under conditions routinely used for differential induction in rats, no differential effect was seen in mice. However differential effects on mouse liver GT activities became obvious when lower doses of phenobarbital (50 mg/kg, daily) were used. When induction of GT activities by 3-MC was studied the enzyme was maximally enhanced (1.5-fold) with a dose of 80 mg/kg. Increasing the dose to 200 mg/kg did not further enhance GT activities. The time course of differential induction of GT activities in livers of C57BL/6 mice is shown in Fig. 1. Phenobarbital treatment (50 mg/kg) selectively enhanced GT_2 activities. In contrast 3-MC administration (80 mg/kg) preferentially enhanced GT_1 activities.

GT and monooxygenase activities in liver microsomes of C57BL/6 and DBA/2 mice, observed after 4 days treatment with the inducing agents, are summarized in Table 2. Monooxygenase reactions showed greater induction by the two agents. In non-responsive DBA/2 mice 3-MC was ineffective as an inducer of both GT and monooxygenase activities, confirming the results of others [15]. However induction by phenobarbital appeared to be unaffected in DBA/2 mice.

Table 2. Differential induction by 3-methylcholanthrene or phenobarbital of liver microsomal UDP-glucuronosyltransferase and monooxygenase activities in C57BL/6 and DBA/2 mice

Enzyme and substrate	Enzyme activity (nmoles/min/mg protein)* C57BL/6 Controls	C57BL/6 3-MC	C57BL/6 Phenobarbital	DBA/2 Controls	DBA/2 3-MC	DBA/2 Phenobarbital
GT activities						
1-Naphthol	24.4 ± 4.2	35.9 ± 3.4† (1.5)	25.4 ± 3.9 (1.1)	18.6 ± 1.7	18.2 ± 1.9 (1.0)	21.8 ± 1.7 (1.2)
4-Methylumbelliferone	63.0 ± 4.8	102.8 ± 8.6† (1.6)	65.2 ± 4.8 (1.0)	48.4 ± 6.4	45.0 ± 5.4 (0.9)	58.4 ± 4.4 (1.2)
3-OH-benzo(a)pyrene	0.56 ± 0.08	0.85 ± 0.07† (1.5)	0.65 ± 0.07 (1.2)	0.34 ± 0.05	0.33 ± 0.06 (1.0)	0.39 ± 0.04 (1.1)
4-OH-biphenyl	31.0 ± 5.0	36.6 ± 5.6 (1.2)	47.1 ± 5.9† (1.5)	26.8 ± 2.9	25.5 ± 2.9 (1.0)	40.7 ± 3.8† (1.5)
Morphine	11.6 ± 1.5	13.5 ± 1.5 (1.2)	16.7 ± 2.3† (1.4)	9.8 ± 2.3	10.6 ± 1.9 (1.1)	15.9 ± 2.3† (1.6)
Monooxygenase						
Benzo(a)pyrene	0.25 ± 0.03	0.78 ± 0.03† (3.1)	0.27 ± 0.03 (1.1)	0.23 ± 0.03	0.22 ± 0.05 (1.0)	0.19 ± 0.05 (0.8)
Aminopyrine	7.4 ± 0.6	11.1 ± 0.05 (1.5)	38.1 ± 0.5† (5.1)	6.4 ± 1.1	6.2 ± 0.8 (1.0)	19.2 ± 0.9† (3.0)

* Data represent the mean ±S.D. of 4 induction experiments. Values in parentheses represent the induction factor, i.e., the ratio between enzyme activity in treated animals and untreated controls.
† $P < 0.01$ compared with controls.

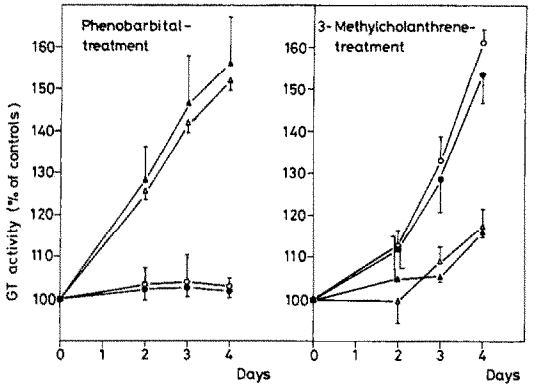

Fig. 1. Differential induction of liver microsomal UDP-glucuronosyltransferase by phenobarbital (50 mg/kg, daily) and 3-MC (80 mg/kg, once). Enzyme activities determined in untreated controls of C57BL/6 mice are given in Table 2. Enzyme substrates are: 1-naphthol (●), 4-methylumbelliferone (○), 4-hydroxybiphenyl (△) and morphine (▲). Data represent the mean ±S.D. ($n = 4$).

Tissue distribution of GT_1 and GT_2 activities. GT_1 activities are clearly detectable in all tissues examined whereas appreciable GT_2 activities were only found in liver and small intestinal mucosa (Table 3). Low but definitely detectable 4-hydroxybiphenyl-GT activities in kidney could be due to either overlapping specificity of this substrate with GT_1 or very low GT_2 levels in kidney. Similar results were obtained with both mouse strains.

Lack of inhibition of GT (1-naphthol as substrate) by morphine. Glucuronidation of 1-naphthol in liver microsones of C57BL/6 mice could not be inhibited by morphine even if the inhibitor was present 385-fold in excess. The lowest 1-naphthol concentration used in the inhibitor study was 7.8 μM. Morphine was added to the assay up to 3 mM. Apparent K_M-values for 1-naphthol-GT and morphine-GT in this tissue were determined as 0.06 and 0.8 mM, respectively. Since they differ only by a factor of 13 the above molar excess should be sufficient to detect inhibition if the two substrates were conjugated at the same active site.

DISCUSSION

Our results suggest functional heterogeneity of distinct GT_1 and GT_2 activities in mice with properties similar to those found in rats: (a) GT_1 activities were preferentially induced by 3-methylcholanthrene whereas GT_2 activities were selectively enhanced by phenobarbital. (b) GT_1 activities were found ubiquitously in mouse tissues whereas GT_2 activities appeared to be restricted to liver and intestinal mucosa. (c) GT_1 (1-naphthol as substrate) could not be inhibited by morphine, a GT_2 substrate, suggesting that the two substrates are conjugated at different active sites.

Liver GT_1 from 3-MC treated rats has been purified to apparent homogeneity with a subunit mol. wt of 54000 [12]. Rabbit antibodies to rat liver GT_1 preferentially precipitated GT_1 activities from solubilized rat liver microsomes. Ouchterlony double-diffusion analysis showed lines of identity between the enzymes from rat liver, rat kidney and mouse liver [30]. Immunological similarity of rat and mouse liver GT was also reported by Burchell [31]. GT_2 activities could be separated from GT_1 activities, although not completely, during purification of the rat liver enzyme [12]. However the properties of the isolated morphine-GT preparation and other GT activities separated from GT_1 activities are insufficiently characterized. Therefore the distinction between GT_1 and GT_2 activities remains operational.

Our findings, together with results of genetic experiments [19], suggest that it is a distinct form of GT, termed GT_1, which is regulated by the Ah locus. Coordinate induction of cytochrome P_1-450 and GT_1 is surprising since two other drug metabolizing enzymes are not linked with the Ah locus: epoxide hydrolase [32] and glutathione *S*-transferase [33]. Parallel induction of GT_1 and AHH activity has also been found in rat hepatoma cell cultures [34]. However there is also evidence for independent control of these two functionally related enzymes [1]. The significance of coordinate induction remains to be elucidated. It may, e.g., greatly facilitate inactivation of polycyclic aromatic compounds and their elimination from the organism. This is supported by the

Table 3. Tissue distribution of UDP-glucuronosyltransferase activities in C57BL/6 and DBA/2 (data in parentheses) mice

Tissue	UDP-glucuronosyltransferase activity* (μmoles/min/g tissue) 1-Naphthol	4-Methyl-umbelliferone	4-Hydroxy-biphenyl	Morphine
Liver	1.60 ± 0.18 (1.27 ± 0.17)	3.69 ± 0.64 (3.49 ± 0.63)	1.80 ± 0.24 (1.60 ± 0.21)	0.54 ± 0.13 (0.74 ± 0.12)
Intestine	0.87 ± 0.13 (0.40 ± 0.13)	0.94 ± 0.43 (0.63 ± 0.11)	0.43 ± 0.15 (0.25 ± 0.04)	0.04 ± 0.01 (0.03 ± 0.01)
Kidney	0.39 ± 0.18 (0.36)	0.47 ± 0.29 (0.30)	0.06 ± 0.02 (0.03)	<0.01 (<0.01)
Lung	0.07 ± 0.02 (0.11)	0.13 ± 0.03 (0.24)	<0.01 (<0.01)	<0.01 (<0.01)
Spleen	0.03 ± 0.01 (0.02)	0.04 ± 0.01 (0.02)	<0.01 (<0.01)	<0.01 (<0.01)
Skin	0.03 ± 0.01 (0.05)	0.05 ± 0.01 (0.09)	<0.01 (<0.01)	n.d. (n.d.)

* Data represent the mean ± S.D. of 4 experiments. n.d. = not determined.

high capacity of GT_1 to conjugate benzo(a)pyrene phenols, in particular 3-hydroxybenzo(a)pyrene which appears to be a typical substrate of GT_1 (Table 2) [12]. Conjugation may prevent further oxidation of phenolic metabolites to ultimate DNA binding species and mutagens [3–6]. It has been shown that benzo(a)pyrene mutagenicity in the Ames test is decreased when cofactors of glucuronidation are present along with the activating monooxygenase system from mouse [4] and rat [6] liver. The effect of glucuronidation was more pronounced with rat liver microsomes from 3-MC-treated rats than with those from phenobarbital-treated animals or untreated controls [6] nicely illustrating the advantage of coordinate induction of both activating and inactivating enzymes. At high benzo(a)pyrene concentrations, however, benzo(a)pyrene mutagenicity was found to be enhanced by the addition of cofactors of glucuronidation [35].

A number of differences in the response of GT_1 to inducing agents have been noted between mice and rats. Response of GT_1 to 3-MC was much weaker in responsive C57BL/6 mice than in Wistar or Sprague–Dawley rats studied previously. Induction factors were about 1.5-fold in mice versus more than 3-fold in rats [12]. On the other hand the response of mouse liver GT_1 activities to phenobarbital appeared to be stronger than in rats. In studies on the regulation of rat liver cytochrome P-450 levels it is well established that 3-MC and phenobarbital induce the synthesis of different forms of cytochrome P-450 [18, 36]. A similar situation may apply to different forms of GT. However it is also known from studies in cell culture that both 3-MC and phenobarbital induce AHH activity [37] as well as GT_1 (4-methylumbelliferone as substrate) [34]. Similarly, higher doses of phenobarbital (apart from its inducing effect of GT_2 activities) may also induce GT_1 activities *in vivo* (Table 1). None of the GT reactions studied so far is inducible by 3-MC in non-responsive DBA/2 mice whereas inducibility of GT activities by phenobarbital is not impaired (Table 2). In contrast to rats bilirubin-GT activity is inducible by 3-MC in liver of C57BL/6 mice but not in DBA/2 mice [38].

Despite these species differences in the response of GT reactions to 3-MC and phenobarbital between mice and rats our results demonstrate that the distinction between GT_1 and GT_2 activities, as defined for rats, can be extended to mice. If the backcross studies with 4-methylumbelliferone-GT [19] are extrapolated to other GT_1 activities it is conceivable that it is GT_1 which is regulated by the Ah locus. Accumulating knowledge on the regulation of drug metabolizing enzymes in responsive and non-responsive inbred strains of mice will help to understand the different genetic predisposition to toxic reactions in these mouse strains and hopefully also in man.

Acknowledgements—We thank Ms. M. Blume, U. Heine, S. Zachmann and cand. med. F. M. Weiss for expert technical assistance and the Deutsche Forschungsgemeinschaft for financial support.

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